Introduction

Cardiac hypertrophy (CH) is a common pathological reaction caused by multiple cardiovascular diseases (CVDs), such as hypertension (1) and pulmonary arterial hypertension (PAH) (2). It is the main pathological basis that ultimately leads to heart failure (3). Right ventricular hypertrophy often occurs in patients with PAH (4,5). The pulmonary vascular remodeling caused by PAH leads to
an increase in vascular resistance, which in turn causes an increase in right ventricular afterload. Left ventricular hypertrophy is common in patients with hypertension (6,7). The pathogenesis of CH is complex and has not yet been fully elucidated. Therefore, identification of novel targets in the pathogenesis of CH is crucial for the prevention and treatment of CH and heart failure.

Calcineurin A (CnA) is one of the catalytic subunits of calcineurin. It is the only Ca²⁺/calmodulin-dependent serine/threonine (Ser/Thr) phosphoprotein phosphatase known thus far (8). In cells, CnA catalyzes the dephosphorylation of nuclear factor of activated T cells (NFAT) protein, which then enters the nucleus (9). It is showed that activation of NFATc2 and its downstream factors can induce CH in mice, and calcineurin-induced CH is blocked in NFATc2 knockout mice (10). Activation of the CnA/NFATc2 pathway promotes cell growth and hypertrophy, and affects the contractile function of cardiomyocytes (11,12). Therefore, the regulation of CnA/NFATc2 pathway may be a novel target for the treatment of CH (13).

MicroRNA (miRNA) is a type of short, conserved endogenous RNA, consisting of about 22 nucleotides (14). MiRNAs efficiently bind to the 3’-untranslated region (UTR) of messenger RNAs (mRNAs). This kind of complementary base pairing can prevent translation or induce mRNA degradation (15). The role of miRNAs, such as miR-26a-5p (16), miR-146a (17), and miR-140-3p (18), in CH is gradually being revealed. Studies have shown that the levels of miR-194 are significantly reduced in children with dilated cardiomyopathy (19). It has also been suggested that miR-194 plays an antioxidant role by targeting MAPK1 in cardiomyocyte ischemia/reperfusion injury (20). Furthermore, the levels of miR-194 are also decreased in impaired renal function caused by hypertension (21). These data suggested that miR-194 may be involved in the occurrence of CH, but its expression characteristics and regulation mechanisms in CH are still unclear.

Therefore, this study analyzed the expression characteristics and effects of miR-194 in a CH cell model induced by isoproterenol (ISO), and explored the mechanisms based on the CnA/NFATc2 pathway. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-1894/rc).

**Methods**

**Cell culture and treatment**

The H9c2 cardiomyocytes (ATCC® CRL-1446) were provided by American Type Culture Collection (ATCC) (USA) and maintained in the RPMI640 complete medium containing 10% fetal bovine serum (FBS; Solarbio), 100 mg of streptomycin/mL (Solarbio), and 100 units of penicillin/mL (Solarbio). The cells were cultured in a 5% CO₂ incubator at 37 °C and 95% humidity. To construct an in vitro CH cell model, cardiomyocyte hypertrophy was induced by ISO (JKN013053, Jingke Chemical Technology Co., Ltd., Shanghai, China). The cells were seeded in a 24-well plate at 1x10⁵ cells per well. The final concentration of ISO in the medium was 10 μmol/L, and the induction time was 48 hours.

**Cell transfection**

To analyze the influence of miR-194 and CnA in cardiomyocytes, miR-194 and/or CnA overexpression and/or silencing was performed. Transfectant sequences were showed in Table 1. Plasmids encoding the full-length human CnA (pcDNA3.1), small interfering (si) CnA, miR-194 mimic, miR-194 inhibitor, and corresponding negative control (NC) were purchased from GenePharma Co., Ltd. (Shanghai, China). The cells were transfected with 100 pmol pcDNA3.1 or 50 nM mimic (7 °C, 5% CO₂, 48 hours) using Lipofectamine™ 2000 transfection reagent (Invitrogen Corporation, Carlsbad, CA, USA). The transfection efficiency was determined by quantitative reverse transcription polymerase chain reaction (RT-qPCR) or Western blot analysis.

**Table 1** Transfectant sequences

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-194 mimic</td>
<td>UGUAAACGCAACUCAGUGGGA</td>
</tr>
<tr>
<td></td>
<td>CACAGUGGAGUUGGCUUCAUU</td>
</tr>
<tr>
<td>Mimic NC</td>
<td>UUCUCCGAACGUGUCACGUTT</td>
</tr>
<tr>
<td>miR-194 inhibitor</td>
<td>UCCACUGAGGUUGCUGUUACA</td>
</tr>
<tr>
<td>Inhibitor NC</td>
<td>UACUCGGACUUUGUCACTAAG</td>
</tr>
<tr>
<td>CnA</td>
<td>CTAGAGAACCCACTGGTTAC</td>
</tr>
<tr>
<td>NC</td>
<td>TAGAAGGCACAGTCGAGG</td>
</tr>
<tr>
<td>siCnA</td>
<td>GAACCGCAAUUUAACGGUC</td>
</tr>
<tr>
<td>siNC</td>
<td>UUCUCGGACGUGUGUCAGGU</td>
</tr>
</tbody>
</table>

NC, negative control; CnA, calcineurin A.
### Table 2 Primer sequences

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-194 (forward)</td>
<td>CTAGTACCTAGAGAACTTTGGAAGACTGTTACAGCTCAGCA</td>
</tr>
<tr>
<td>miR-194 (reverse)</td>
<td>AGCTTGCTGAGCTGTAACAGTCTTCAAAGGTTCCTCTAGGTA</td>
</tr>
<tr>
<td>U6 (forward)</td>
<td>ACCCTGAGAAATACCCTCACAT</td>
</tr>
<tr>
<td>U6 (reverse)</td>
<td>GACGACTGAGCCCTGATG</td>
</tr>
<tr>
<td>ANP (forward)</td>
<td>GGCTCCTTCTCCATCACCAAA</td>
</tr>
<tr>
<td>ANP (reverse)</td>
<td>TGTTATCTTCGGTACCG</td>
</tr>
<tr>
<td>can (forward)</td>
<td>CACTTCTTGTCTGTAAGCCG</td>
</tr>
<tr>
<td>can (reverse)</td>
<td>AAGGCAATTGATCCCAAGTT</td>
</tr>
<tr>
<td>GAPDH (forward)</td>
<td>ACCACAGTCCATGCCATACCA</td>
</tr>
<tr>
<td>GAPDH (reverse)</td>
<td>TCCACCACCTGTGGTGT</td>
</tr>
</tbody>
</table>

CnA, calcineurin A; ANP, atrial natriuretic peptide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

### Assessment of CH

Cells were seeded in a 24-well plate at $5 \times 10^3$ cells per well. After 24 hours, the cells were fixed with 4% paraformaldehyde (P6148, Sigma-Aldrich, St. Louis, MO, USA). After permeabilization with 0.1% Triton X-100, cells were stained with DAPI and phalloidin (Life Technology, St. Louis, MO, USA) for 10 and 40 minutes, respectively, in the dark. After washing and removing the reagents, LSM800 confocal microscope (Zeiss, Germany) was used for imaging. According to the fluorescence range, ImageJ was applied to calculate the cell surface area.

### RT-qPCR

H9c2 cells were harvested and RT-qPCR was performed for the detection of mRNA expression. Briefly, the total RNA in the cells was extracted using Trizol Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Using the Primescript RT Reagent kit (Takara, Shiga, Japan), each total RNA sample (1 μg) was subjected to reverse transcription reaction to obtain the cDNA template. The qPCR amplification was performed with SYBR Green reagent (Takara) using the ABI 7500 fast real-time PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95 °C for 10 seconds, followed by 40 cycles of 95 °C for 10 seconds, 50 °C for 30 seconds, and 72 °C for 30 seconds. The expression of mRNA was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta C_T}$ method.

Total miRNA was extracted using miRNeasy Mini kit (GE Healthcare, USA), and the cDNA was generated using the TaqMan miRNA reverse transcription kit (DBI Bioscience, Germany). The TaqMan miRNA kit (DBI Bioscience, Germany) was applied to measure the expression levels of the miRNA, which was normalized to U6 using the $2^{-\Delta\Delta C_T}$ method. Primer sequences were showed in Table 2.

### Western blot

H9c2 cells were harvested and incubated with RIPA lysis solution on ice for 30 minutes. Cell lysate samples were centrifuged for 20 minutes at 4 °C, 12,000 xg to obtain the supernatant. The concentration of the total proteins in the supernatant was determined using the BCA kit (Beyotime Biotechnology, Jiangsu, China). In addition, proteins in the cytoplasm and nucleus were separated using an Ambion PARISTM Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed for the separation of proteins. After being transferred to a polyvinylidene fluoride membrane, proteins were blocked with 5% skimmed milk for 2 hours at room temperature. Membranes were incubated with rabbit anti-primary antibodies (1:1,000) overnight at 4 °C. The primary antibodies used are as follows: anti-atrial natriuretic peptide (ANP; 1:1,000, sc-515701, Santa Cruz, CA, USA), anti-CnA (1:1,000, ab52761, Abcam, Cambridge, MA, USA), anti-NFAT c2 (1:1,000, sc-7296), anti-GAPDH (1:1,000, ab8245), and anti-lamin B1 (1:1,000, ab229025). GAPDH was used as the control for cytoplasmic protein or total protein in the cells. Lamin B1 was used as the control for proteins in the nucleus. Thereafter, Tris-buffered saline/0.1% Tween (TBST) solution was used to wash the membranes twice. Horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody (1:2,000, ab6721, Abcam, Cambridge, MA, USA) was used to incubate the membranes for 2 hours at 37 °C. Membranes were washed 3 times with TBST and the protein blots were visualized using enhanced chemiluminescence (ECL) (Solarbio). Image analysis software IPP6.0 was applied for the analysis of gray intensity.

### Dual luciferase report

The 3’-UTR sequence of wild-type (wt-) CnA mRNA was
amplified to the downstream site of the pGL4 luciferase vector (Promega, Madison, WI, USA). The rapid site-directed mutagenesis kit (D0206, Beyotime, Shanghai, China) was used to generate the mutated (mut-) CnA mRNA 3'-UTR luciferase plasmid, 50 nM miR-194 mimic or miR-194 NC, and 150 ng of Renilla luciferase plasmid (Beyotime, Shanghai, China) were transfected into cells via Lipofectamine™ 2000. The cells were then incubated at 37 °C for 36 hours. The dual luciferase reporter gene detection kit (Promega, Madison, WI, USA) was used to detect luciferase activity according to the manufacturer’s protocol. All data were normalized to Renilla luciferase activity.

**Statistical analysis**

All experiments were performed independently 3 times. Each index was measured 3 times. Data are presented as mean ± standard deviation (SD) and analyzed using SPSS19.0 software (SPSS Inc., Chicago, IL, USA). The Student’s t-test was used for comparison between two groups. For comparison among at least three groups, one-way analysis of variance (ANOVA) was applied. A P value <0.05 was considered statistically significant.

**Results**

After ISO-induced hypertrophy of H9c2 cells, the levels of miR-194 expression decreases and the CnA/NFATc2 pathway was activated

H9c2 cells were seeded in a 24-well plate at a density of 3×10^4/well. After 24 hours, 1 µg of wt-CnA mRNA 3’-UTR or mut-CnA mRNA 3’-UTR luciferase plasmid, 50 nM miR-194 mimic or miR-194 NC, and 150 ng of Renilla luciferase plasmid (Beyotime, Shanghai, China) were transfected into cells via Lipofectamine™ 2000. The cells were then incubated at 37 °C for 36 hours. The dual luciferase reporter gene detection kit (Promega, Madison, WI, USA) was used to detect luciferase activity according to the manufacturer’s protocol. All data were normalized to Renilla luciferase activity.

**MiR-194 targeting inhibits CnA protein expression**

Sequence prediction analysis revealed the binding sites between miR-194 and the 3'-UTR of the CnA mRNA (Figure 2A). Dual luciferase reporter assays demonstrated that when miR-194 mimic and wt-CnA mRNA 3'-UTR were transfected into cells, the relative luciferase activity was significantly inhibited. When the CnA mRNA 3'-UTR was mutated, it could not bind to miR-194, and the luciferase activity remained unchanged compared to wt-CnA (Figure 2B). This suggested that miR-194 directly targeted and bound to the CnA mRNA 3'-UTR. In addition, overexpression of miR-194 was performed by transfecting cells with the miR-194 mimic (Figure 2C). When the levels of miR-194 were elevated, the expression of the CnA protein was significantly suppressed (P<0.05; Figure 2D), suggesting that miR-194 inhibits the expression of CnA protein by targeting the 3'-UTR of the CnA mRNA.

Decreasing the expression of miR-194 causes cardiomyocyte hypertrophy through the CnA/NFATc2 pathway

To analyze the effects of miR-194 and CnA on cardiomyocyte hypertrophy, H9c2 cells were divided into 4 groups as follows: NC, miR-194 inhibitor, siCnA, and miR-194 inhibitor + siCnA. Western blot analysis revealed that when the levels of miR-194 were suppressed, the levels of CnA protein were significantly elevated. The CnA protein levels in the siCnA group were significantly lower than that of the NC group (P<0.05; Figure 3A,3B). This not only indicated that the transfection experiment was successful, but also further verified that miR-194 could inhibit the expression of CnA protein. Consistent with the expression levels of the CnA protein, both the reduction of miR-194 and the overexpression of CnA protein promoted the nuclear translocation of NFATc2 protein. Moreover, interference with CnA blocked the entry of NFATc2 into the nucleus caused by miR-194 downregulation (P<0.05; Figure 3C-3E). The cell surface area was increased in the presence of the miR-194 inhibitor, while interference
with CnA decreased the cell surface area. When the expression of CnA was interfered with, transfection of the miR-194 inhibitor could no longer increase the cell surface area (P<0.05; Figure 3F). This further verified that cardiomyocyte hypertrophy caused by a reduction in miR-194 expression is inseparable from the CnA/NFATc2 pathway.

**Overexpression of miR-194 alleviates hypertrophy of H9c2 cells and activation of CnA/NFATc2 pathway induced by ISO**

To further analyze whether miR-194 can alleviate the myocardial hypertrophy induced by ISO, rescue experiments were conducted. H9c2 cells were divided into

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**Figure 1** In H9c2 cells induced by ISO, the expression of miR-194 decreases and the CnA/NFATc2 pathway is activated. (A) The effects of ISO on cardiomyocyte hypertrophy (surface area) were assessed by immunofluorescence staining (×400). (B) The effects of ISO on the expression of ANP mRNA, a marker of cardiac hypertrophy in cardiomyocytes. (C) The effects of ISO on the expression of ANP protein expression. (D) The effects of ISO on the expression of miR-194. (E) The effects of ISO on the expression of CnA mRNA. (F-I) The effects of ISO on the expression of CnA protein and NFATc2 protein nuclear translocation. *, P<0.05 vs. control group. ISO, isoproterenol; miR, microRNA; CnA, calcineurin A; NFATc2, nuclear factor of activated T cells c2; ANP, atrial natriuretic peptide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
Figure 2 Targeting miR-194 inhibits calcineurin A protein expression. (A) The binding sites of miR-194 and the 3'-UTR of CnA mRNA. (B) Dual luciferase report assays verified the targeted binding of miR-194 to the 3'-UTR of CnA mRNA. (C) The levels of miR-194 in cells transfected with miR-194 NC and miR-194 mimic. (D) The expression of CnA protein in cells after overexpression of miR-194. *, P<0.05 vs. NC group. miR, microRNA; CnA, calcineurin A; UTR, untranslated region; WT, wild-type; mut, mutant; NC, negative control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Discussion

CVD is characterized by high morbidity, disability, and mortality. Approximately 17 million people worldwide die from CVD each year, accounting for about one-third of patients who died of disease. It is estimated that by 2030, the number of deaths due to CVD every day will reach 65,000 (22,23). CVD, such as hypertension and myocardial infarction, can cause CH, and ultimately affect the ejection function of the heart. However, the mechanisms of CH remain to be fully elucidated.

The regulatory mechanisms of post-transcriptional non-coding RNAs (ncRNAs) is a new research focus. The post-transcriptional regulation of miRNAs directly affects the expression of proteins and thus participates in the process
Figure 3 Decreasing the expression of miR-194 causes cardiomyocyte hypertrophy through the CnA/NFATc2 pathway. (A) The levels of miR-194 in cells transfected with miR-194 NC and miR-194 inhibitor. (B) The expression levels of CnA protein in each group. (C-E) The nuclear translocation of the NFATc2 protein in each group of cells. (F) Comparison of the surface area in each group of cells by immunofluorescence staining (×400). *, P<0.05 vs. NC group; #, P<0.05 vs. miR-194 inhibitor group; +, P<0.05 vs. siCnA group. miR, microRNA; CnA, calcineurin A; NFATc2, nuclear factor of activated T cells c2; NC, negative control; si, small interfering; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

of CH, which makes miRNAs a potential target for the treatment of CH (24). For example, miR-26a-5p directly inhibits the expression of glycogen synthase kinase 3 beta (GSK3β), elevates the expression of ANP and other proteins, and increases the cell surface area (25). In ISO-induced CH, increasing the levels of miR-29a alleviates CH through targeted inhibition of peroxisome proliferator-activated receptor δ (26). In this study, ISO was successfully used to simulate in vitro CH, resulting in a 1.8-fold increase in the surface area of cardiomyocytes. After induction, the levels of miR-194 in the cells were significantly reduced. Furthermore, the levels of CnA mRNA and protein in the cells were elevated, and the downstream NFATc2 protein entered the nucleus. In addition, the dual luciferase reporter experiments showed that miR-194 directly targets and binds to the 3′-UTR of CnA mRNA. Overexpression of miR-194 significantly inhibited the expression of the CnA protein. This study demonstrated the alterations in miR-194 expression and the CnA/NFATc2 pathway in cardiomyocyte hypertrophy. Further research is warranted to elucidate the precise mechanisms involved.

While homeostasis of Ca^{2+} is the key to maintaining the normal morphology and function of cardiomyocytes, it is also involved in the process of β-adrenaline-induced CH (27). The stimulation of β-adrenaline affects the oscillation frequency of Ca^{2+}, thereby activating CnA (8). This in turn activates the nuclear translocation of the NFATc2 protein (28). Indeed, activation of NFATc2 plays a crucial role in ISO-induced CH (8). The CnA/NFATc2 pathway can be targeted and inhibited by miRNAs. For example, Zhang et al. (29) reported that miR-137 inhibits NFAT by reducing the expression of CnA, thereby regulating the de-differentiation and proliferation of smooth muscle cells. CnA is also targeted and regulated by miR-206-3p and miR-381-3p, thereby affecting the transcription levels of inducible nitric oxide synthase (iNOS), which regulates NFATc2 and affects muscle cell function (30,31). MiR-30 also protects the morphology
and function of podocytes by inhibiting the CnA/NFATc2 pathway (32). To analyze the effects of miR-194/CnA/NFATc2 on cardiomyocytes, cells models in which miR-194 and/or CnA expression were inhibited were constructed. The results showed that suppression of miR-194 increased the levels of CnA protein, enhanced nuclear translocation of NFATc2, and increased the cell surface area. Interference of CnA expression blocked the inhibitory effects of miR-194 on cardiomyocyte hypertrophy. This suggested that reduction of miR-194 expression causes cardiomyocyte hypertrophy by activating the CnA/NFATc2 pathway.

Previous clinical studies have shown that the levels of miR-194 in the plasma of children with idiopathic dilated cardiomyopathy is significantly reduced (19). Furthermore, in vitro studies have demonstrated that increasing the levels of miR-194 protected the human renal tubular epithelial cells from hypoxia/reperfusion injury (35). After the heart is damaged, fibrosis and hypertrophy can occur simultaneously (36). The hypoxia-induced mitogenic factor promotes the occurrence of CH by aggravating the inflammatory response, and the levels of intracellular fibrin inevitably increase in the process (37). A study has shown that miR-194 inhibits the expression of the Runx1 gene at the post-transcriptional level, thereby regulating the AKT pathway and inhibiting renal fibrosis (38). To further analyze the role of miR-194 in CH, rescue experiments were conducted. The data revealed that after elevating the levels of miR-194, the increased cells surface area induced by ISO was significantly inhibited. In addition, the increase in CnA protein expression caused by ISO and the nuclear translocation of NFATc2 were also alleviated. This suggested that ISO may promote the transcription of CnA and increase CnA protein levels, which in turn activates NFATc2 and induces nuclear translocation,
thereby inducing cardiomyocyte hypertrophy. Therefore, increasing the levels of miR-194 may target and inhibit CnA expression at the post-transcriptional level, and alleviate cardiomyocyte hypertrophy.

Conclusions

In summary, increasing the expression of miR-194 can alleviate CH by targeting can and inhibiting the CnA/NFATc2 pathway. The characteristics of miR-194 in CH require further clinical research. Furthermore, the inhibitory effects of miR-194 on CH and the CnA/NFATc2 pathway warrant further verification through in vivo experiments.

Acknowledgments

Funding: The study was funded by General Project of Chongqing Natural Science Foundation (No. cstc2021jcyj-msxmX0307).

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-1894/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-1894/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-1894/coif). All authors report that the study was funded by General Project of Chongqing Natural Science Foundation (No. cstc2021jcyj-msxmX0307). The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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